

# A VARIANT OF LAV VIRUSES

## 5 BACKGROUND OF THE INVENTION

10 The present invention relates to a virus capable of inducing lymphadenopathies (hereinafter "LAS") and acquired immuno-depressive syndromes (hereinafter "AIDS"), to antigens of this virus, particularly in a purified form, and to a process for producing these antigens, particularly antigens of the envelope of this virus. The invention also relates to polypeptides, whether glycosylated or not, produced by the virus and to DNA sequences which code for such polypeptides. The invention further relates to cloned DNA sequences hybridizable to genomic RNA and DNA of the lymphadenopathy associated virus (hereinafter "LAV") of this invention and to processes for their preparation and their use. 20 The invention still further relates to a stable probe including a DNA sequence which can be used for the detection of the LAV virus of this invention or related viruses or DNA proviruses in any medium, particularly biological, and in samples containing any of them.

25 An important genetic polymorphism has been recognized for the human retrovirus which is the cause of AIDS and other diseases like LAS, AIDS-related complex (hereinafter "ARC") and probably some encephalopathies (for review, see Weiss, 1984). Indeed all of the isolates, analyzed until now, have had distinct restriction maps, even those recovered at the same place and time [Benn et al., 1985]. Identical restriction maps have only been observed for the first two isolates which were designated LAV [Alizon et al., 1984] and human T-cell 30 lymphotropic virus type 3 (hereinafter "HTLV-3") [Hahn et al., 1984] and which appear to be exceptions. The

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genetic polymorphism of the AIDS virus was better assessed after the determination of the complete nucleotide sequence of LAV [Wain-Hobson et al., 1985], HTLV-3 [Ratner et al., 1985 ; Muesing et al., 1985] and a third isolate designated AIDS-associated retrovirus (hereinafter "ARV 2") [Sanchez-Pescador et al., 1985]. In particular, it appeared that, besides the nucleic acid variations responsible for the restriction map polymorphism, isolates could differ significantly at the protein level, especially in the envelope (up to 13 % of difference between ARV and LAV), by both amino acids substitutions and reciprocal insertions-deletions [Rabson and Martin, 1985].

Nevertheless, such differences did not go so far as to destroy the immunological similarity of such isolates as evidenced by the capabilities of their similar proteins, (e.g., core proteins of similar nature, such as the p25 proteins, or similar envelope glycoproteins, such as the 110-120 kD glycoproteins) to immunologically cross-react. Accordingly, the proteins of any of said LAV viruses can be used for the in vitro detection of antibodies induced in vivo and present in biological fluids obtained from individuals infected with the other LAV variants. Therefore, these viruses are grouped together as a class of LAV viruses (hereinafter "LAV-1 viruses").

#### SUMMARY OF THE INVENTION

In accordance with this invention, a new virus has been discovered that is responsible for diseases clinically related to AIDS and that can be classified as a LAV-1 virus but that differs genetically from known LAV-1 viruses to a much larger extent than the known LAV-1 viruses differ from each other. The new virus

is basically characterized by the cDNA sequence which is shown in Figures 7A to 7I, and this new virus is hereinafter generally referred to as "LAV<sub>ELI</sub>".

Also in accordance with this invention, variants of the new virus are provided. The RNAs of these variants and the related cDNAs derived from said RNAs are hybridizable to corresponding parts of the cDNA of LAV<sub>ELI</sub>. The DNA of the new virus also is provided, as well as DNA fragments derived therefrom hybridizable with the genomic RNA of LAV<sub>ELI</sub>, such DNA and DNA fragments particularly consisting of the cDNA or cDNA fragments of LAV<sub>ELI</sub> or of recombinant DNAs containing such cDNA or cDNA fragments.

DNA recombinants containing the DNA or DNA fragments of LAV<sub>ELI</sub> or its variants are also provided. It is of course understood that fragments which would include some deletions or mutations which would not substantially alter their capability of also hybridizing with the retroviral genome of LAV<sub>ELI</sub> are to be considered as forming obvious equivalents of the DNA or DNA fragments referred to hereinabove.

Cloned probes are further provided which can be made starting from any DNA fragment according to the invention, as are recombinant DNAs containing such fragments, particularly any plasmids amplifiable in procaryotic or eucaryotic cells and carrying said fragments. Using cloned DNA containing a DNA fragment of LAV<sub>ELI</sub> as a molecular hybridization probe - either by marking with radionucleotides or with fluorescent reagents - LAV virion RNA may be detected directly, for example, in blood, body fluids and blood products (e.g., in antihemophylic factors such as Factor VIII concentrates). A suitable method for achieving such detection comprises immobilizing LAV<sub>ELI</sub> on a support (e.g., a nitrocellulose filter), disrupting the virion and

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hybridizing with a labelled (radiolabelled or solid fluorescent- or enzyme-labelled) probe. Such an approach has already been developed for Hepatitis B virus in peripheral blood (according to Scotto J. et al. Hepatology (1983), 2, 379-384).

Probes according to the invention can also be used for rapid screening of genomic DNA derived from the tissue of patients with LAV related symptoms to see if the proviral DNA or RNA present in their tissues is related to LAV<sub>221</sub>. A method which can be used for such screening comprises the following steps: extraction of DNA from tissue, restriction enzyme cleavage of said DNA, electrophoresis of the fragments and Southern blotting of genomic DNA from tissues and subsequent hybridization with labelled cloned LAV proviral DNA. Hybridization in situ can also be used. Lymphatic fluids and tissues and other non-lymphatic tissues of humans, primates and other mammalian species can also be screened to see if other evolutionary related retroviruses exist. The methods referred to hereinabove can be used, although hybridization and washings would be done under non-stringent conditions.

The DNA according to the invention can be used also for achieving the expression of LAV viral antigens for diagnostic purposes, as well as for the production of a vaccine against LAV. Fragments of particular advantage in that respect will be discussed later. The methods which can be used are multifold:

a) DNA can be transfected into mammalian cells with appropriate selection markers by a variety of techniques, such as calcium phosphate precipitation, polyethylene glycol, protoplast-fusion, etc.

b) DNA fragments corresponding to genes can be cloned into expression vectors for E. coli, yeast or mammalian cells and the resultant proteins purified.

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capable of cleaving it at specific sites. By way of examples may be mentioned the enzyme of Staphylococcus aureus V8,  $\alpha$ -chymotrypsine, "mouse sub-maxillary gland protease" marketed by the Boehringer company, Vibrio alginolyticus chemovar lophagus collagenase, which specifically recognizes the peptides Gly-Pro, Gly-Ala, etc.

Other features of this invention will appear in the following disclosure of data obtained starting from LAV<sub>ELI</sub>, in relation to the drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- 15 - Figs. 1A and 1B provide comparative restriction maps of the genomes of LAV<sub>ELI</sub> as compared to LAV<sub>MAL</sub> (Applicants' related new LAV virus which is the subject of their copending application, filed herewith) and LAV<sub>BRU</sub> (a known LAV isolate deposited at the Collection Nationale des Cultures de Micro-organismes (hereinafter "CNCM") of the Pasteur Institute, Paris, France under No. I-232 on July 15, 1983) ;
- 20 - Fig. 2 shows comparative maps setting forth the relative positions of the open reading frames of the above genomes ;
- 25 - Figs. 3A-3F (also designated generally hereinafter "fig. 3") indicate the relative correspondance between the proteins (or glycoproteins) encoded by the open reading frames, whereby amino acid residues of protein sequences of LAV<sub>ELI</sub> are in vertical alignment with corresponding amino acid residues (numbered) of corresponding or homologous proteins or glycoproteins of LAV<sub>BRU</sub>, as well as LAV<sub>MAL</sub> and ARV 2;
- 30 - Figs. 4A-4B (also designated generally hereinafter "fig. 4") provide tables quantitating the sequence divergence between homologous proteins of LAV<sub>BRU</sub>, LAV<sub>ELI</sub>
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and LAV<sub>MAL</sub> ;

- Fig. 5 shows diagrammatically the degree of divergence of the different virus envelope proteins ;

5 - Figs. 6A and 6B ("Fig. 6" when consulted together) render apparent the direct repeats which appear in the proteins of the different AIDS virus isolates.

- Figs. 7A-7I show the full nucleotidic sequences of LAV<sub>ELI</sub>.

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#### DETAILED DESCRIPTION OF THE INVENTION

##### **CHARACTERIZATION AND MOLECULAR CLONING OF AN AFRICAN ISOLATE.**

15 The different AIDS virus isolates concerned are designated by three letters of the patients name, LAV<sub>BRU</sub> referring to the prototype AIDS virus isolated in 1983 from a French homosexual patient with LAS and thought to have been infected in the USA in the preceding years [Barré-Sinoussi et al., 1983]. LAV<sub>ELI</sub> was recovered in 1983 from a 24-year old woman with AIDS 20 from Zaire. Related LAV<sub>MAL</sub> was recovered in 1985 from a 7-year old boy from Zaire. Recovery and purification of the LAV<sub>ELI</sub> virus were performed according to the method disclosed in European Patent Application 84 401834/138 667 filed on September 9, 1984.

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LAV<sub>ELI</sub> is indistinguishable from the previously characterized isolates by its structural and biological properties in vitro. Virus metabolic labelling and immune precipitation by patient ELI sera, as well as reference sera, showed that the proteins of LAV<sub>ELI</sub> had 30 the same molecular weight (hereinafter "MW") as, and cross-reacted immunologically with those of, prototype AIDS virus (data not shown) of the LAV-1 class.

Reference is again made to European Application 178 978 and International Application PCT/EP 35

85/00548 as concerns the purification, mapping and sequencing procedures used herein. See also the discussion under the headings "Experimental Procedures" and "Significance of the Figures" hereinafter.

Primary restriction enzyme analysis of LAV<sub>ELI</sub> genome was done by southern blot with total DNA derived from acutely infected lymphocytes, using cloned LAV<sub>BRU</sub> complete genome as probe. Overall cross-hybridization was observed under stringent conditions, but the restriction profile of the Zairian isolate was clearly different. Phage lambda clones carrying the complete viral genetic information were obtained and further characterized by restriction mapping and nucleotide sequence analysis. A Clone (hereinafter "E-H12") was derived from LAV<sub>ELI</sub> infected cells and contained an integrated provirus with 5' flanking cellular sequences but a truncated 3' long terminal repeat (hereinafter "LTR").

Figure 1B gives a comparison of the restriction maps derived from the nucleotide sequences of LAV<sub>ELI</sub>, LAV<sub>MAL</sub> and prototype LAV<sub>BRU</sub>, as well as from three other Zairian isolates (hereinafter "Z1", "Z2", and "Z3" respectively) previously mapped for seven restriction enzymes [Benn et al., 1985]. Despite this limited number, all of the profiles are clearly different (out of the 23 sites making up the map of LAV<sub>BRU</sub>, only seven are present in all six maps presented), confirming the genetic polymorphism of the AIDS virus. No obvious relationship is apparent between the five Zairian maps, and all of their common sites are also found in LAV<sub>BRU</sub>.

#### Conservation of the genetic organization.

The genetic organization of LAV<sub>ELI</sub> as deduced from the complete nucleotide sequences of its cloned genome is identical to that found in other isolates, i.e., 5'gag-pol-central region-env-F3'. Most noticeable is the conservation of the "central region" (fig. 2),



located between the pol and env genes, which is composed of a series of overlapping open reading frames (hereinafter "orf") previously designated Q, R, S, T, and U in the ovine lentivirus visna [Sonigo et al., 1985]. The product of orf S (also designated "tat") is implicated in the transactivation of virus expression [Sodroski et al., 1985 ; Arya et al., 1985] ; the biological role of the product of orf Q (also designated "sor" or "orf A") is still unknown [Lee et al., 1986 ; Kang et al., 1986]. Of the three other orfs, R, T, and U, only orf R is likely to be a seventh viral gene, for the following reasons : the exact conservation of its relative position with respect to Q and S (fig. 2), the constant presence of a possible splice acceptor and of a consensus AUG initiator codon, its similar codon usage with respect to viral genes, and finally the fact that the variation of its protein sequence within the different isolates is comparable to that of gag, pol and Q (see fig. 4).

Also conserved are the sizes of the U3, R and U5 elements of the LTR (data not shown), the location and sequence of their regulatory elements such as TATA box and AATAAA polyadenylation signal, and their flanking sequences, i.e., primer binding site (hereinafter "PBS") complementary to 3' end of tRNA<sup>LYS</sup> and polypurine tract (hereinafter "PPT"). Most of the genetic variability within the LTR is located in the 5' half of U3 (which encodes a part of orf F) while the 3' end of U3 and R, which carry most of the cis-acting regulatory elements, promoter, enhancer and trans-activating factor receptor [Rosen et al., 1985], as well as the U5 element, are well-conserved.

Overall, it clearly appears that this Zairian isolate, LAV<sub>ELI</sub>, is the same type of retrovirus as the previously sequenced isolates of American or European

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origin.

### Variability of the viral proteins.

Despite their identical genetic organization, the LAV<sub>ELI</sub> and LAV<sub>MAL</sub> shows substantial differences in the primary structure of their proteins. The amino acid sequences of LAV<sub>ELI</sub> and LAV<sub>MAL</sub> proteins are presented in figures 3A-3F, aligned with those of LAV<sub>BRU</sub> and ARV 2. Their divergence was quantified as the percentage of amino acids substitutions in two-by-two alignments (Fig. 4). The number of insertions and deletions that had to be introduced in each of these alignments has also been scored.

Three general observations can be made. First, the protein sequences of the LAV<sub>ELI</sub> and LAV<sub>MAL</sub> are more divergent from LAV<sub>BRU</sub> than are those of HTLV-3 and ARV 2 (Fig. 4A) ; similar results are obtained if ARV 2 is taken as reference (not shown). The range of genetic polymorphism between isolates of the AIDS virus is considerably greater than previously observed. Second, our two sequences confirm that the envelope is more variable than the gag and pol genes. Here again, the relatively small difference observed between the env of LAV<sub>BRU</sub> and HTLV-3 appears as an exception. Third, the mutual divergence of the LAV<sub>ELI</sub> and LAV<sub>MAL</sub> (Fig. 4B) is comparable to that between LAV<sub>BRU</sub> and either of them; as far as we can extrapolate from only three sequenced isolates from the USA and Europe and two (LAV<sub>ELI</sub> and LAV<sub>MAL</sub>) from Africa, this is indicative of a wider evolution of the AIDS virus in Africa.

gag and pol : Their greater degree of conservation compared to the envelope is consistent with their encoding important structural or enzymatic activities. Of the three mature gag proteins, the p25 which was the first recognized immunogenic protein of LAV [Barré-Sinoussi et al., 1983] is also the better conserved

(fig. 3). In gag and pol, differences between isolates are principally due to point mutations, and only a small number of insertional or deletional events is observed.

Among these, we must note the presence in the overlapping part of gag and pol of LAV<sub>BRU</sub> of an insertion of 12 amino acids (AA) which is encoded by the second copy of a 36 bp direct repeat present only in this isolate and in HTLV-3. This duplication was omitted because of a computing error in the published sequence of LAV<sub>BRU</sub> (position 1712, Wain-Hobson et al., 1985) but was indeed present in the HTLV-3 sequences [Ratner et al., 1985 ; Muesing et al., 1985].

env : Three segments can be distinguished in the envelope glycoprotein precursor [Allan et al., 1985 ; Montagnier et al., 1985 ; DiMarzoVeronese et al., 1985]. The first is the signal peptide (positions 1-33 in fig. 3), and its sequence appears as variable ; the second segment (pos. 34-530) forms the outer membrane protein (hereinafter "OMP" or "gp110") and carries most of the genetic variations, and in particular almost all of the numerous reciprocal insertions and deletions ; the third segment (531-877) is separated from the OMP by a potential cleavage site following a constant basic stretch (Arg-Glu-Lys-Arg) and forms the transmembrane protein (hereinafter "TMP" or "gp 41") responsible for the anchorage of the envelope glycoprotein in the cellular membrane. A better conservation of the TMP than the OMP has also been observed between the different murine leukemia viruses (hereinafter "MLV") [Koch et al., 1983] and could be due to structural constraints.

From the alignment of figure 3 and the graphical representation of the envelope variability shown in figure 5, we clearly see the existence of conserved domains, with little or no genetic variation, and hypervariable domains, in which even the alignment

of the different sequences is very difficult, because of the existence of a large number of mutations and of reciprocal insertions and deletions. We have not included the sequence of the envelope of the HTLV-3 isolate since it is so close to that of LAV<sub>BRU</sub> (cf. fig. 4), even in the hypervariable domains, that it did not add anything to the analysis. While this graphical representation will be refined by more sequence data, the general profile is already apparent, with three hypervariable domains (Hyl, 2 and 3) all being located in the OMP and separated by three well-conserved stretches (residues 37-130, 211-289, and 488-530 of fig. 3 alignment) probably associated with important biological functions.

In spite of the extreme genetic variability, the folding pattern of the envelope glycoprotein is probably constant. Indeed the position of virtually all of the cysteine residues is conserved within the different isolates (fig. 3 and 5), and the only three variable cysteines fall either in the signal peptide or in the very C-terminal part of the TMP. The hypervariable domains of the OMP are bounded by conserved cysteines, suggesting that they may represent loops attached to the common folding pattern. Also the calculated hydropathic profiles [Kyte and Doolittle, 1982] of the different envelope proteins are remarkably conserved (not shown).

About half of the potential N-glycosylation sites, Asn-X-Ser/Thr, found in the envelopes of the Zairian isolates map to the same positions in LAV<sub>BRU</sub> (17/26 for LAV<sub>ELI</sub> and 17/28 for LAV<sub>MAL</sub>). The other sites appear to fall within variable domains of env, suggesting the existence of differences in the extent of envelope glycosylation between different isolates.

Other viral proteins : Of the three other identified viral proteins, the p27 encoded by orf F, 3' of env

[Allan et al., 1985b] is the most variable (fig. 4). The proteins encoded by orfs Q and S of the central region are remarkable by their absence of insertions/deletions. Surprisingly, a high frequency of amino acids substitutions, comparable to that observed in env, is found for the product of orf S (trans-activating factor). On the other hand, the protein encoded by orf Q is no more variable than gag. Also noticeable is the lower variation of the proteins encoded by the central regions of LAV<sub>ELI</sub> and LAV<sub>MAL</sub>.

With the availability of the complete nucleotide sequence from five independant isolates, some general features of the AIDS virus' genetic variability are now emerging. Firstly, its principal cause is point mutations which very often result in amino acid substitutions and which are more frequent in the 3' part of the genome (orf S, env and orf F). Like all RNA viruses, the retroviruses are thought to be highly subject to mutations caused by errors of the RNA polymerases during their replication, since there is no proofreading, of this step [Holland et al., 1982 ; Steinhauer and Holland, 1986].

Another source of genetic diversity is insertions/deletions. From the figure 3 alignments, insertional events seem to be implicated in most of the cases, since otherwise deletions should have occurred in independant isolates at precisely the same locations. Furthermore, upon analyzing these insertions, we have observed that they most often represent one of the two copies of a direct repeat (fig. 6). Some are perfectly conserved like the 36 bp repeat in the gag-pol overlap of LAV<sub>BRU</sub> (fig. 6-a) ; others carry point mutations resulting in amino acid substitutions, and as a consequence, they are more difficult to observe, though

clearly present, in the hypervariable domains of env (cf. fig. 6-g and -h). As noted for point mutations, env gene and orf F also appear as more susceptible to that form of genetic variation than the rest of the genome. 5 The degree of conservation of these repeats must be related to their date of occurrence in the analyzed sequences : the more degenerated, the more ancient. A very recent divergence of LAV<sub>BRU</sub> and HTLV-3 is suggested by the extremely low number of mismatched AA between 10 their homologous proteins. However, one of the LAV<sub>BRU</sub> repeats (located in the Hyl domain of env, fig. 6-f) is not present in HTLV-3, indicating that this generation of tandem repeats is a rapid source of genetic diversity. We have found no traces of such a phenomenon, even 15 when comparing very closely related viruses, such as the Mason-Pfizer monkey virus (hereinafter "MPMV") [Sonigo et al., 1986], and an immunosuppressive simian virus (hereinafter "SRV-1") [Power et al., 1986]. Insertion or deletion of one copy of a direct repeat have been occasionally reported in mutant retroviruses [Shimotohno and 20 Temin, 1981 ; Darlix, 1986], but the extent to which we observe this phenomenon is unprecedented. The molecular basis of these duplications is unclear, but could be the "copy-choice" phenomenon, resulting from the diploidy of 25 the retroviral genome [Varmus and Swanstrom, 1984 ; Clark and Mak, 1983]. During the synthesis of the first-strand of the viral DNA, jumps are known to occur from one RNA molecule to another, especially when a break or a stable secondary structure is present on the template; 30 an inaccurate re-initiation on the other RNA template could result in the generation (or the elimination) of a short direct repeat.

Genetic variability and subsequent antigenic 35 modifications have often been developed by micro-organisms as a means for avoiding the host's immune

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response, either by modifying their epitopes during the course of the infection, as in trypanosomes [Borst and Cross, 1982], or by generating a large repertoire of antigens, as observed in influenza virus [Webster et al., 1982]. As the human AIDS virus is related to animal lentiviruses [Sonigo et al., 1985 ; Chiu et al., 1985], its genetic variability could be a source of antigenic variation, as can be observed during the course of the infection by the ovine lentivirus visna [Scott et al., 1979 ; Clements et al., 1980] or by the equine infectious anemia virus (hereinafter "EIAV") [Montelaro et al., 1984]. However, a major discrepancy with these animal models is the extremely low, and possibly nonexistent, neutralizing activity of the sera of individuals infected by the AIDS virus, whether they are healthy carriers, displaying minor symptoms, or afflicted with AIDS [Weiss et al., 1985 ; Clavel et al., 1985]. Furthermore, even for the visna virus the exact role of antigenic variation in the pathogenesis is unclear [Thormar et al., 1983 ; Lutley et al., 1983]. We rather believe that genetic variation represents a general selective advantage for lentiviruses by allowing an adaptation to different environments, for example by modifying their tissue or host tropisms. In the particular case of the AIDS virus, rapid genetic variations are tolerated, especially in the envelope. This could allow the virus to become adapted to different "micro-environments" of the membrane of their principal target cells, namely the T4 lymphocytes. These "micro-environments" could result from the immediate vicinity of the virus receptor to polymorphic surface proteins, differing either between individuals or between clones of lymphocytes.

#### Conserved domains in the AIDS virus envelope

Since the proteins of most of the isolates are

antigenically cross-reactive, the genotypic differences do not seem to affect the sensitivity of actual diagnostic tests, based upon the detection of antibodies to the AIDS virus and using purified virions as antigens. They nevertheless have to be considered for the development of the "second-generation" tests, that are expected to be more specific, and will use smaller synthetic or genetically-engineered viral antigens. The identification of conserved domains in the highly immunogenic envelope glycoprotein and the core structural proteins (gag) is very important for these tests. The conserved stretch found at the end of the OMP and the beginning of the TMP (490-620, fig. 3) could be a good candidate, since a bacterial fusion protein containing this domain was well-detected by AIDS patients' sera [Chang et al., 1985].

The envelope, specifically the OMP, mediates the interaction between a retrovirus and its specific cellular receptor [DeLarco and Todaro, 1976 ; Robinson et al., 1980]. In the case of the AIDS virus, in vitro binding assays have shown the interaction of the envelope glycoprotein gp110 with the T4 cellular surface antigen [McDougal et al., 1986], already thought to be closely associated with the virus receptor [Klatzmann et al., 1984 ; Dagleish et al., 1984]. Identification of the AIDS virus envelope domains that are responsible for this interaction (receptor-binding domains) appears to be fundamental for understanding of the host-viral interactions and for designing a protective vaccine, since an immune response against these epitopes could possibly elicit neutralizing antibodies. As the AIDS virus receptor is at least partly formed of a constant structure, the T4 antigen, the binding site of the envelope is unlikely to be exclusively encoded by domains undergoing drastic genetic changes between



isolates, even if these could be implicated in some kind of an "adaptation". One or several of the conserved domains of the OMP (residues 37-130, 211-289, and 488-530 of fig. 3 alignment), brought together by the folding of the protein, must play a part in the virus-receptor interaction, and this can be explored with synthetic or genetically-engineered peptides derived from these domains, either by direct binding assays or indirectly by assaying the neutralizing activity of specific antibodies raised against them.

#### African AIDS viruses

Zaire and the neighboring countries of Central Africa are considered as an area endemic with the AIDS virus infection, and the possibility that the virus has emerged in Africa has become a subject of intense controversy (see Norman, 1985). From the present study, it is clear that the genetic organization of Zairian isolates is the same as that of american isolates, thereby indicating a common origin. The very important sequence differences observed between the proteins are consistent with a divergent evolutionary process. In addition, the two African isolates are mutually more divergent than the American isolates already analyzed ; as far as that observation can be extrapolated, it suggests a longer evolution of the virus in Africa and is also consistent with the fact that a larger fraction of the population is exposed than in developed countries.

A novel human retrovirus with morphology and biological properties (cytopathogenicity, T4 tropism) similar to those of LAV, but nevertheless clearly genetically and antigenically distinct from it, was recently isolated from two patients with AIDS originating from Guinea Bissau, West-Africa [Clavel et al., 1986]. In neighboring Senegal, the population

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was seemingly exposed to a retrovirus also distinct from LAV but apparently non-pathogenic [Barin et al., 1985 ; Kanki et al., 1986]. Both of these novel African retroviruses seem to be antigenically related to the simian T-cell lymphotropic virus (hereinafter "STLV-III") shown to be widely present in healthy African green monkeys and other simian species [Kanki et al. 1985]. This raises the possibility of a large group of African primate lentiviruses, ranging from the apparently non-pathogenic simian viruses to the LAV-type viruses. Their precise relationship will only be known after their complete genetic characterization, but it is already very likely that they have evolved from a common progenitor. The important genetic variability we have observed between isolates of the AIDS virus in Central Africa is probably a hallmark of this entire group and may account for the apparently important genetic divergence between its members (loss of cross-antigenicity in the envelopes). In this sense, the conservation of the tropism for the T4 lymphocytes suggests that it is a major advantage acquired by these retroviruses.

#### EXPERIMENTAL PROCEDURES

##### **Virus isolation**

LAV<sub>ELI</sub> was isolated from the peripheral blood lymphocytes of the patient as described [Barré-Sinoussi et al., 1983]. Briefly, the lymphocytes were fractionated and co-cultivated with phytohaemagglutinin-stimulated normal human lymphocytes in the presence of interleukin 2 and anti-alpha interferon serum. Viral production was assessed by cell-free reverse transcriptase (hereinafter "RT") activity assay in the cultures and by electron microscopy.

##### **Molecular cloning**

Normal donor lymphocytes were acutely infected

( $10^4$  cpm of RT activity/ $10^6$  cells) as described [Barré-Sinoussi et al., 1983], and total DNA was extracted at the beginning of the RT activity peak. A lambda library using the L47-1 vector [Loenen and Brammar, 1982] was constructed by partial HindIII digestion of the DNA as already described [Alizon et al., 1984]. About  $5 \cdot 10^5$  plaques for LAV<sub>ELI</sub>, obtained by *in vitro* packaging (Amersham), were plated on *E. coli* LA101 and screened *in situ* under stringent conditions, using the 9 kb SacI insert of the clone lambda J19 [Alizon et al., 1984] carrying most of the LAV<sub>BRU</sub> genome as probe. Clones displaying positive signals were plaque-purified and propagated on *E. coli* C600 recBC, and the recombinant phage E-H12 carrying the complete genetic information of LAV<sub>ELI</sub> was further characterized by restriction mapping.

#### Nucleotide sequence strategy

Viral fragments derived from E-H12 were sequenced by the dideoxy chain terminator procedure [Sanger et al., 1977] after "shotgun" cloning in the M13mp8 vector [Messing and Viera, 1982] as previously described [Sonigo et al., 1985]. The viral genome of LAV<sub>ELI</sub> is 9176 nucleotides long as shown in figures 7A-7I. Each nucleotide of LAV<sub>ELI</sub> was determined from more than 5 independent clones on average.

#### SIGNIFICANCE OF THE FIGURES

**Figure 1** contains an analysis of AIDS virus isolates, showing:

A/ Restriction maps of the inserts of phage lambda clones derived from cells infected with LAV<sub>ELI</sub> (E-H12) and with LAV<sub>MAL</sub> (hereinfter "M-H11"). The schematic genetic organization of the AIDS virus has been drawn above the maps. The LTRs are indicated by solid boxes. Restriction sites are indicated as follows:  
A:Aval; B:BamHI; Bg:BgIII; E:EcoRI; H:HindIII; Hc:HincII; K:KpnI; N:NdeI; P:PstI; S:SacI; and X:XbaI.

B/ A comparison of the sites for seven  
restriction enzymes in six isolates : the prototype AIDS  
virus LAV<sub>BRU</sub>, LAV<sub>MAL</sub> and LAV<sub>ELI</sub> ; and Z1, Z2 and Z3.  
Restriction sites are represented by the following  
symbols vertically aligned with the symbols in fig. 1a: ● :  
BgIII; \* :EcoRI; ▽ :HincII; ▼ :HindIII; ◆ :KpnI; ◇ :NdeI;  
and o: SacI.

25 **Figure 3** shows an alignment of the protein sequences of  
four AIDS virus isolates. Isolate LAV<sub>BRU</sub> [Wain-Hobson et  
al., 1985] is taken as reference ; only differences with  
LAV<sub>BRU</sub> are noted for ARV 2 [Sanchez-Pescador et al.,  
1985] and the two Zairian isolates LAV<sub>MAL</sub> and LAV<sub>ELI</sub>. A  
30 minimal number of gaps (-) were introduced in the  
alignments. The NH<sub>2</sub>-termini of p25<sup>gag</sup> and p18<sup>gag</sup> are  
indicated [Sanchez-Pescador, 1985]. The potential  
cleavage sites in the envelope precursor [Allan et al.,  
1985a ; diMarzoVeronese, 1985] separating the signal  
peptide (hereinafter "SP"), OMP and TMP are indicated as  
35 vertical arrows ; conserved cysteines are indicated by

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(identical AA in the 4 sequences) to 16 (4 different AA). This type of representation has previously been used in a compilation of the AA sequence of immunoglobulins variable regions [Wu and Kabat, 1970]. Vertical arrows indicate the cleavage sites ; asterisks represent potential N-glycosylation sites (N-X-S/T) conserved in all four isolates ; black triangles represent conserved cysteine residues. Black lozanges mark the three major hydrophobic domains: OMP, TMP and SP; and the hyper-variable domains: Hyl, 2 and 3.

**Figure 6** shows the direct repeats in the proteins of different AIDS virus isolates. These examples are derived from the aligned sequences of gag (a, b), F (c,d) and env (e, f, g, h) shown in figure 3. The two elements of the direct repeat are boxed, while degenerated positions are underlined.

Figures 7A-7I show the complete cDNA sequence of LAV<sub>ELI</sub> of this invention.

The invention thus pertains more specifically to the proteins, glycoproteins and other polypeptides including the polypeptidic structures shown in the figures 1-7. The first and last amino acid residues of these proteins, glycoproteins and polypeptides carry numbers computed from a first amino acid of the open-reading frames concerned, although these numbers do not correspond exactly to those of the LAV<sub>ELI</sub> proteins concerned, rather to the corresponding proteins of the LAV<sub>BRU</sub> or sequences shown in figs. 3A, 3B and 3C. Thus a number corresponding to a "first amino acid residue" of a LAV<sub>ELI</sub> protein corresponds to the number of the first amino-acyl residue of the corresponding LAV<sub>BRU</sub> protein which, in any of figs. 3A, 3B or 3C, is in direct alignment with the corresponding first amino acid of the LAV<sub>ELI</sub> protein. Thus the sequences concerned can be read from figs. 7A-7I to the extent where they do not appear

with sufficient clarity from Figs. 3A-3F.

The preferred protein sequences of this invention extend between the corresponding "first" and "last" amino acid residues. Also preferred are the protein(s)- or glycoprotein(s)-portions including part of the sequences which follow :

OMP or gp110 proteins, including precursors :  
1 to 530

OMP or gp110 without precursor :  
34-530

Sequence carrying the TMP or gp41 protein :  
531-877, particularly  
680-700

well conserved stretches of OMP :  
37-130,  
211-289 and  
488-530

well conserved stretch found at the end of the OMP and the beginning of TMP :  
490-620.

Proteins containing or consisting of the "well conserved stretches" are of particular interest for the production of immunogenic compositions and (preferably in relation to the stretches of the env protein) of vaccine compositions against the LAV-1 viruses.

The invention concerns more particularly all the DNA fragments which have been more specifically referred to in the drawings and which correspond to open reading frames. It will be understood that one skilled in the art will be able to obtain them all, for instance by cleaving an entire DNA corresponding to the complete genome of LAV<sub>ELI</sub>, such as by cleavage by a partial or complete digestion thereof with a suitable restriction enzyme and by the subsequent recovery of the relevant fragments. The DNA disclosed above can be resorted to

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forming higher eucaryotic cells particularly mammalian cells. Preferably, any of the abovesaid sequences are placed under the direct control of a promoter contained in said vectors and recognized by the polymerases of said cells, such that the first nucleotide codons expressed correspond to the first triplets of the above-defined DNA sequences. Accordingly, this invention also relates to the corresponding DNA fragments which can be obtained from the genome of LAV<sub>ELI</sub> or its cDNA by any appropriate method. For instance, such a method comprises cleaving said LAV<sub>ELI</sub> genome or its cDNA by restriction enzymes preferably at the level of restriction sites surrounding said fragments and close to the opposite extremities respectively thereof, recovering and identifying the fragments sought according to sizes, if need be checking their restriction maps or nucleotide sequences (or by reaction with monoclonal antibodies specifically directed against epitopes carried by the polypeptides encoded by said DNA fragments), and further if need be, trimming the extremities of the fragment, for instance by an exonucleolytic enzyme such as Bal31, for the purpose of controlling the desired nucleotide sequences of the extremities of said DNA fragments or, conversely, repairing said extremities with Klenow enzyme and possibly ligating the latter to synthetic polynucleotide fragments designed to permit the reconstitution of the nucleotide extremities of said fragments. Those fragments may then be inserted in any of said vectors for causing the expression of the corresponding polypeptide by the cell transformed therewith. The corresponding polypeptide can then be recovered from the transformed cells, if need be after lysis thereof, and purified by methods such as electrophoresis. Needless to say, all conventional methods for performing these operations can be resorted to.

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mammalian species can also be screened to see if other evolutionary related retrovirus exist. The methods referred to hereinabove can be used, although hybridization and washings would be done under non-stringent conditions.

The DNAs or DNA fragments according to the invention can be used also for achieving the expression of viral antigens of LAV<sub>ELI</sub> for diagnostic purposes.

The invention relates generally to the polypeptides themselves, whether synthesized chemically, isolated from viral preparations or expressed by the different DNAs of the invention, particularly by the ORFs or fragments thereof in appropriate hosts, particularly procaryotic or eucaryotic hosts, after transformation thereof with a suitable vector previously modified by the corresponding DNAs.

More generally, the invention also relates to any of the polypeptide fragments (or molecules, particularly glycoproteins having the same polypeptidic backbone as the polypeptides mentioned hereinabove) bearing an epitope characteristic of a protein or glycoprotein of LAV<sub>ELI</sub>, which polypeptide or molecule then has N-terminal and C-terminal extremities respectively either free or, independently from each other, covalently bonded to amino acids other than those which are normally associated with them in the larger polypeptides or glycoproteins of the LAV virus, which last mentioned amino acids are then free or belong to another polypeptidic sequence. Particularly, the invention relates to hybrid polypeptides containing any of the epitope-bearing-polypeptides which have been defined more specifically hereinabove, recombined with other polypeptides fragments normally foreign to the LAV proteins, having sizes sufficient to provide for an increased immunogenicity of the epitope-bearing-polypeptide yet, said

foreign polypeptide fragments either being immunogenically inert or not interfering with the immunogenic properties of the epitope-bearing-polypeptide.

Such hybrid polypeptides, which may contain from 5 up to 150, even 250 amino acids, usually consist of the expression products of a vector which contained ab initio a nucleic acid sequence expressible under the control of a suitable promoter or replicon in a suitable host, which nucleic acid sequence had however beforehand been modified by insertion therein of a DNA sequence encoding said epitope-bearing-polypeptide.

Said epitope-bearing-polypeptides, particularly those whose N-terminal and C-terminal amino acids are free, are also accessible by chemical synthesis according to technics well known in the chemistry of proteins.

The synthesis of peptides in homogeneous solution and in solid phase is well known. In this respect, recourse may be had to the method of synthesis in homogeneous solution described by Houbenweyl in the work entitled "Methoden der Organischen Chemie" (Methods of Organic Chemistry) edited by E. WUNSCH., vol. 15-I and II, THIEME, Stuttgart 1974. This method of synthesis consists of successively condensing either the successive amino acids in twos, in the appropriate order or successive peptide fragments previously available or formed and containing already several amino-acyl residues in the appropriate order respectively. Except for the carboxyl and aminogroups which will be engaged in the formation of the peptide bonds, care must be taken to protect beforehand all other reactive groups borne by these amino-acyl groups or fragments. However, prior to the formation of the peptide bonds, the carboxyl groups are advantageously activated, according to methods well known in the synthesis of peptides. Alternatively, recourse may be had to coupling reactions

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bringing into play conventional coupling reagents, for instance of the carbodiimide type, such as 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide. When the amino acid group used carries an additional amine group (e.g., lysine) or another acid function (e.g., glutamic acid), these groups may be protected by carbobenzoxy or t-butyloxycarbonyl groups, as regards the amine groups, or by t-butylester groups, as regards the carboxylic groups. Similar procedures are available for the protection of other reactive groups. For example, an -SH group (e.g., in cysteine) can be protected by an acetamidomethyl or paramethoxybenzyl group.

In the case of a progressive synthesis, amino acid by amino acid, the synthesis starts preferably with the condensation of the C-terminal amino acid with the amino acid which corresponds to the neighboring amino-acyl group in the desired sequence and so on, step by step, up to the N-terminal amino acid. Another preferred technique which can be used is that described by R.D. Merrifield in "Solid Phase Peptide Synthesis" [J. Am. Chem. Soc., 45, 2149-2154]. In accordance with the Merrifield process, the first C-terminal amino acid of the chain is fixed to a suitable porous polymeric resin, by means of its carboxylic group, the amino group of the amino acid then being protected, for example by a t-butyloxycarbonyl group. When the first C-terminal amino acid is thus fixed to the resin, the protective group of the amine group is removed by washing the resin with an acid, i.e., trifluoroacetic acid, when the protective group of the amine group is a t-butyloxycarbonyl group. Then, the carboxylic group of the second amino acid, which is to provide the second amino-acyl group of the desired peptidic sequence, is coupled to the deprotected amine group of the C-terminal amino acid

fixed to the resin. Preferably, the carboxyl group of this second amino acid has been activated, for example by dicyclohexyl-carbodiimide, while its amine group has been protected, for example by a t-butyloxycarbonyl group. The first part of the desired peptide chain, which comprises the first two amino acids, is thus obtained. As previously, the amine group is then de-protected, and one can further proceed with the fixing of the next amino-acyl group and so forth until the whole peptide sought is obtained. The protective groups of the different side groups, if any, of the peptide chain so formed can then be removed. The peptide sought can then be detached from the resin, for example by means of hydrofluoric acid, and finally recovered in pure form from the acid solution according to conventional procedures.

As regards the peptide sequences of smallest size bearing an epitope or immunogenic determinant, and more particularly those which are readily accessible by chemical synthesis, it may be required, in order to increase their *in vivo* immunogenic character, to couple or "conjugate" them covalently to a physiologically acceptable and non-toxic carrier molecule. By way of examples of carrier molecules or macromolecular supports which can be used for making the conjugates according to the invention can be mentioned natural proteins, such as tetanic toxoid, ovalbumin, serum-albumins, hemocyanins, etc. Synthetic macromolecular carriers, for example polysines or poly(D-L-alanine)-poly(L-lysine)s, can be used too. Other types of macromolecular carriers that can be used, which generally have molecular weights higher than 20,000, are known from the literature. The conjugates can be synthesized by known processes such as are described by Frantz and Robertson in "Infect. and Immunity", 33, 193-198 (1981) and by P.E. Kauffman in

"Applied and Environmental Microbiology", October 1981  
V 1. 42, No. 4, pp. 611-614. For instance, the following  
coupling agents can be used : glutaric aldehyde, ethyl  
chloroformate, water-soluble carbodiimides such  
as(N-ethyl-N'(3-dimethylamino-propyl) carbodiimide,  
HCl), diisocyanates, bis-diazobenzidine, di- and  
trichloro-s-triazines, cyanogen bromides and  
benzaquinone, as well as the coupling agents mentioned  
in "Scand. J. Immunol.", 1978, vol. 8, pp. 7-23  
(Avrameas, Ternynck, Guesdon).

Any coupling process can be used for bonding  
one or several reactive groups of the peptide, on the  
one hand, and one or several reactive groups of the  
carrier, on the other hand. Again coupling is advanta-  
geously achieved between carboxyl and amine groups  
carried by the peptide and the carrier or vice-versa in  
the presence of a coupling agent of the type used in  
protein synthesis, e.g., 1-ethyl-3-(3-dimethylaminopro-  
pyl)-carbodiimide, N-hydroxybenzotriazole, etc. Coupling  
between amine groups respectively borne by the peptide  
and the carrier can also be made with glutaraldehyde,  
for instance, according to the method described by  
BOQUET, P. et al. (1982) Molec. Immunol., 19, 1441-1549,  
when the carrier is hemocyanin.

The immunogenicity of epitope-bearing-peptides  
can also be reinforced by oligomerisation thereof, for  
example in the presence of glutaraldehyde or any other  
suitable coupling agent. In particular, the invention  
relates to the water soluble immunogenic oligomers thus  
obtained, comprising particularly from 2 to 10 monomer  
units.

The glycoproteins, proteins and other polypep-  
tides (generally designated hereinafter as "antigens" of  
this invention) whether obtained by methods, such as are  
disclosed in the earlier patent applications referred to

above, in a purified state from LAV<sub>ELI</sub> virus preparations or - as concerns more particularly the peptides - by chemical synthesis, are useful in processes for the detection of the presence of anti-LAV antibodies in biological media, particularly biological fluids such as sera from man or animal, particularly with a view of possibly diagnosing LAS or AIDS.

Particularly the invention relates to an in vitro process of diagnosis making use of an envelope glycoprotein or of a polypeptide bearing an epitope of this glycoprotein of LAV<sub>ELI</sub> for the detection of anti-LAV antibodies in the serums of persons who carry them. Other polypeptides - particular those carrying an epitope of a core protein - can be used too.

A preferred embodiment of the process of the invention comprises :

- depositing a predetermined amount of one or several of said antigens in the cups of a titration microplate ;
- introducing increasing dilutions of the biological fluid, to be diagnosed (e.g., blood serum, spinal fluid, lymphatic fluid, and cephalo-rachidian fluid), into these cups ;
- incubating the microplate ;
- washing carefully the microplate with an appropriate buffer ;
- adding into the cups specific labelled antibodies directed against blood immunoglobulins and
- detecting the antigen-antibody-complex formed, which is then indicative of the presence of LAV antibodies in the biological fluid.

Advantageously the labelling of the anti-immunoglobulin antibodies is achieved by an enzyme selected from among those which are capable of hydrolysing a substrate, which substrate undergoes a modification of its radiation-absorption, at least within a



predetermined band of wavelenghts. The detection of the substrate, preferably comparatively with respect to a control, then provides a measurement of the potential risks, or of the effective presence, of the disease.

Thus, preferred methods of immuno-enzymatic and also immunofluorescent detections, in particular according to the ELISA technique, are provided. Titrations may be determinations by immunofluorescence or direct or indirect immuno-enzymatic determinations. Quantitative titrations of antibodies on the serums studied can be made.

The invention also relates to the diagnostic kits themselves for the in vitro detection of antibodies against the LAV virus, which kits comprise any of the polypeptides identified herein and all the biological and chemical reagents, as well as equipment, necessary for performing diagnostic assays. Preferred kits comprise all reagents required for carrying out ELISA assays. Thus preferred kits will include, in addition to any of said polypeptides, suitable buffers and anti-human immunoglobulins, which anti-human immunoglobulins are labeled either by an immunofluorescent molecule or by an enzyme. In the last instance, preferred kits also comprise a substrate hydrolysable by the enzyme and providing a signal, particularly modified absorption of a radiation, at least in a determined wavelength, which signal is then indicative of the presence of antibody in the biological fluid to be assayed with said kit.

It can of course be of advantage to use several proteins or polypeptides not only of LAV<sub>ELI</sub>, but also of LAV<sub>MAL</sub> together with homologous proteins or polypeptides of earlier described viruses, such as LAV<sub>BRU</sub>, HTLV-3, ARV 2, etc.

The invention also relates to vaccine compositions whose active principle is to be constituted by any

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of the antigens, i.e., the hereinabove disclosed polypeptides of LAV<sub>ELI</sub>, particularly the purified gp110 or immunogenic fragments thereof, fusion polypeptides or oligopeptides in association with a suitable pharmaceutically or physiologically acceptable carrier. A first type of preferred active principle is the gp110 immunogen of said immunogens. Other preferred active principles to be considered in that fields consist of the peptides containing less than 250 amino acid units, preferably less than 150, particularly from 5 to 150 amino acid residues, as deducible for the complete genome of LAV<sub>ELI</sub> and even more preferably those peptides which contain one or more groups selected from Asn-X-Thr and Asn-X-Ser as defined above. Preferred peptides for use in the production of vaccinating principles are peptides (a) to (f) as defined above. By way of example, there may be mentioned that suitable dosages of the vaccine compositions are those which are effective to elicit antibodies *in vivo*, in the host, particularly a human host. Suitable doses range from 10 to 500 micrograms of polypeptide, protein or glycoprotein per kg, for instance 50 to 100 micrograms per kg.

The different peptides according to this invention can also be used themselves for the production of antibodies, preferably monoclonal antibodies specific for the respective different peptides. For the production of hybridomas secreting said monoclonal antibodies, conventional production and screening methods can be used. These monoclonal antibodies, which themselves are part of the invention, provide very useful tools for the identification and even determination of relative proportions of the different polypeptides or proteins in biological samples, particularly human samples containing LAV or related viruses.

The invention further relates to the hosts

(procaryotic or eucaryotic cells) which are transformed by the above mentioned recombinants and which are capable of expressing said DNA fragments.

5 Finally the invention also concerns vectors for transforming eucaryotic cells of human origin, particularly lymphocytes, the polymerase of which are capable of recognizing the LTRs of LAV. Particularly said vectors are characterized by the presence of a LAV  
10 LTR therein, said LTR being then active as a promoter enabling the efficient transcription and translation in a suitable host of a DNA insert coding for a determined protein placed under its controls.

Needless to say, the invention extends to all  
15 variants of genomes and corresponding DNA fragments (ORFs) having substantially equivalent properties, all of said genomes belonging to retroviruses which can be considered as equivalents of LAV<sub>ELI</sub>. It must be understood that the claims which follow are also intended to  
20 cover all equivalents of the products (glycoproteins, polypeptides, DNAs, etc.) whereby an equivalent is a product, e.g., a polypeptide, which may distinguish from a product defined in any of said claims, say through one or several amino acids, while still having substantially  
25 the same immunological or immunogenic properties. A similar rule of equivalency shall apply to the DNAs, it being understood that the rule of equivalency will then be tied to the rule of equivalency pertaining to the polypeptides which they encode.

30 It will also be understood that all the literature referred to hereinbefore and hereinafter and all patent applications and patents not specifically identified herein but which form counterparts of those specifically designated herein, must be considered as  
35 incorporated herein by reference.

It should further be mentioned that the

invention further relates to immunogenic compositions that contain preferably one or more of the polypeptides, which are specifically identified above and which have the amino acid sequences of LAV<sub>ELI</sub> that have been identified, or peptidic sequences corresponding to previously defined LAV proteins. In this respect, the invention relates more particularly to the particular polypeptides which have the sequences corresponding more specifically to the LAV<sub>BRU</sub> sequences which have been referred to earlier, i.e., the sequences extending between the following first and last amino acids, of the LAV<sub>BRU</sub> proteins themselves, i.e., the polypeptides having sequences contained in the LAV<sub>BRU</sub> OMP or LAV<sub>BRU</sub> TMP or sequences extending over both, particularly those extending from between the following positions of the amino acids included in the env open reading frame of the LAV<sub>BRU</sub> genome,

1-530  
34-530  
and more preferably  
531-877, particularly 680-700,  
37-130  
211-289  
488-530  
490-620.

These different sequences can be used for any of the above defined purposes and in any of the compositions which have been disclosed.

Finally the invention also relates to the different antibodies which can be formed specifically against the different peptides which have been disclosed herein, particularly to the monoclonal antibodies which recognize them specifically. The corresponding hybridomas which can be formed starting from spleen cells previously immunized with such peptides which are fused

with appropriate myeloma cells and selected according to standard procedures also form part of the invention.

5 Phage  $\lambda$  clone E-H12 derived from LAV<sup>ELI</sup> infected cells has been deposited at the CNCM under No. I-550 on May 9, 1986. Phage clone M-H11 derived from LAV<sup>MAL</sup> infected cells has been deposited at the CNCM under No. I-551 on May 9, 1986.

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